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Method of preventing bacteriophage infection of bacterial cultures

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1

METHOD OF PREVENTING BACTERIOPHAGE INFECTION OF BACTERIAL CULTURES

FIELD OF INVENTION

The present invention relates to the field of bacterial
5 cultures which are used industrially in the manufacturing of
e.g. food products or useful metabolite products. In particu-
lar there is provided modified bacterial strains which, when
cultivated in a selected substrate material, are not suscep-
tible to attack by bacteriophages, and have retained their
10 capability of being metabolically active.

TECHNICAL BACKGROUND AND PRIOR ART

Production failures of bacterial cultures caused by bacterio-
phage infection are considered to be one of the major prob-
lems in industrial use of bacterial cultures. Bacteriophages
15 have been found for many of the bacterial strains used in the
industry, such as species of lactic acid bacteria e.g. *Lacto-*
coccus spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pedio-*
coccus spp., *Streptococcus* spp., *Propionibacterium* spp.,
Bifidobacterium spp., *Staphylococcus* spp. or *Micrococcus* spp.
20 Furthermore, bacteriophage infections are also well known in
other industrially useful species such as *Bacillus* spp.,
Enterobacteriaceae spp. including *E. coli*, *Corynebacterium*
spp. *Actinomycetes* spp. and *Brevibacterium* spp.

In the food industry lactic acid bacterial starter cultures
25 are widely used for food fermentations. It appears that among
members of the lactic acid bacteria *Lactococcus* spp. are most
devastated by bacteriophage infections. A factor, which leads
to frequent bacteriophage infections in lactic acid bacterial
starter cultures is the fact that the fermentation conditions
30 in the food industry including the dairy industry are gen-
erally non-sterile. Thus, it has not yet been possible to

eliminate bacteriophage contamination under these industrial conditions.

The lytic development of phages involves adsorption of the phages to the host cell surface, injection of phage DNA into the cell, synthesis of phage proteins, replication of phage DNA, assembly of progeny phages and release of progeny from the host. Cell-mediated mechanisms of interference with any of these events can prevent a phage infection. The ability of bacterial cultures to resist bacteriophage infection during industrial use depends to a large extent on host strain characteristics affecting one or more of the above mechanisms.

Thus, it has been shown that natural bacteriophage resistance or defense mechanisms exist in bacterial strains which ensure a certain level of protection against bacteriophage attack. These natural defense mechanisms include phage adsorption inhibition, prevention of phage DNA penetration, restriction of phage DNA and abortive infection.

The prevention of productive contact between phages and bacterial cells due to altered cell surface receptors for phages greatly reduces the ability of the phage to attack the cells. Adsorption of the phages to the cell surface is not always sufficient for the translocation of the phage DNA. It has been shown that host specific cell membrane proteins are involved in the prevention of phage DNA penetration.

Restriction/modification is a mechanism that operates by the cooperation of two enzyme systems, a DNA-cleaving restriction enzyme and a DNA-modifying enzyme, usually a methylase. The mechanism functions by cleaving the phage DNA, as it enters the cell.

Abortive phage infection is described as a mechanism that interferes with the phage development after phage expression

has begun. This may eventually lead to a reduced level or termination of the production of viable phage progeny.

However, like many other traits of bacterial strains which
5 are important for industrial performance, the above described
natural phage defense mechanisms have been shown to be
unstable characteristics, as they may be mediated by plas-
mids. Furthermore, these defense mechanisms are often phage
specific, i.e. they are only active against a limited range
10 of bacteriophage types. Accordingly, the prior art is not
aware of a general and stably maintained host cell associated
resistance mechanism against bacteriophage infection.

Based on the above natural defense mechanisms, the industry
has designed and implemented strategies to possibly reduce
15 bacteriophage infection of bacterial cultures including
starter cultures for the fermentation of dairy products.
Currently used strategies include the use of mixed starter
cultures and alternate use of strains having different phage
susceptibility profiles (strain rotation).

20 Traditionally, starter cultures in the dairy industry are
mixtures of lactic acid bacterial strains. The complex compo-
sition of mixed starter cultures ensures that a certain level
of resistance to phage attack is present. However, repeated
subculturing of mixed strain cultures leads to unpredictable
25 changes in the distribution of individual strains and event-
ually undesired strain dominance. This in turn may lead to
increased susceptibility to phage attack and risk of fermen-
tation failures.

Rotation of selected bacterial strains which are sensitive to
30 different phages is another approach to limit phage develop-
ment. However, it is difficult and cumbersome to identify and
select a sufficient number of strains having different phage
type profiles to provide an efficient and reliable rotation
program. In addition, the continuous use of strains requires
35 careful monitoring for new infectious phages and the need to

quickly substitute a strain which is infected by the new bacteriophage by a resistant strain. In manufacturing plants where large quantities of bulk starter cultures are made ahead of time, such a quick response is usually not possible.

- 5 Studies have shown that a reduced growth capacity of a bacterial culture such as a proteinase-deficient lactic acid bacterium results in reduced phage proliferation (Richardson et al., 1984). However, such bacterial strains are still growing and are thus still susceptible to attack by phages.
- 10 Thus, the industry is not in the possession of any reliable strategy to secure that bacterial cultures used for industrial manufacturing of food products or other products are resistant against attack by bacteriophages. Furthermore, none of the currently used strategies prevent infections by any
- 15 bacteriophages and none of these strategies are capable of precluding that bacteriophages, by a mutational event, circumvent the resistance mechanisms of the bacterial culture strains.

- It is therefore a significant objective of the present invention to provide a method of preventing bacteriophage infection of bacterial cultures which are used in the manufacturing of food products and other products, wherein the cultures are completely resistant to bacteriophage attack.
- 20

25 SUMMARY OF THE INVENTION

- Accordingly, the invention provides in a first aspect a method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not
- 30 susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of replication or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,

5 (ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the material under conditions
10 where the culture is metabolically active,

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

The invention pertains in another aspect to the use of a
15 culture as obtained in the method of the invention as a starter culture in the preparation of a product selected from the group consisting of a starter distillate, a product for cheese flavouring, a food product and a feed product.

DETAILED DISCLOSURE OF THE INVENTION

20 Thus, in its broadest aspect the invention provides a method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, said method comprising the
25 steps (i) to (iii) as mentioned above. As used herein, the expression "modifying a substrate material" is used interchangeably with the term "fermentation" and relates to any aerobic or anaerobic breakdown of organic compounds by a bacterial culture with the production of an end product. In
30 addition, it will be appreciated that the expression "metabolically active" refers to the capability of the bac-

terial culture to convert a substrate material such as e.g. milk or a sugar.

In the present context, the expression "not susceptible to attack by bacteriophages" includes the capability of a host cell to be metabolically active even though a bacteriophage adsorbs to the host cell surface and injects its DNA into the host cell.

In accordance with the invention, the method comprises in one aspect the isolation of a bacterial strain which is not capable of replication or protein synthesis in a specific defined substrate material, but is capable of metabolically modifying said material. Evidently, it was a very surprising finding that it is possible to provide such non-proliferating bacterial strains which are unable to grow in specific defined substrate materials, but which have retained their capability of being metabolically active. As used herein, the expression "non-proliferating bacterial strain" relates to a bacterial strain which is incapable to multiply.

In a particularly useful embodiment of the present method the above specific substrate material is limited with respect to at least one nutrient compound that is required by the bacterial strain for replication or protein synthesis. Such compounds include amino acids or nitrogenous bases such as purine and pyrimidine bases.

Thus, the growth of the bacterial strain is prevented due to the incapability of the strains to synthesize the specific compound with respect to which the substrate is limited. Such a mutant strain which has lost such a capability is also referred to in the art as an "auxotrophic strain". Therefore, in preferred embodiments, the bacterial strain is a mutant strain being auxotrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication or protein synthesis.

The substrate material used in the method of the invention may in a further useful embodiment contain at least one compound that inhibits the replication or the protein synthesis of the bacterial strain. Examples of such compounds include chloramphenicol and erythromycin which affect the ribosomes of the bacterial cell and thus inhibit protein synthesis.

In accordance with the invention, the propagation of the selected strain prior to its use in the present method requires a medium wherein the strain is capable of replicating to obtain a culture of said strain. It is assumed that a medium containing the specific compound which the mutant is unable to synthesize, will restore the capability of the mutant to grow, i.e. capability of replication and/or protein synthesis.

In a further step of the method according to the invention, the above obtained bacterial culture is added to the above substrate material and kept under conditions where the culture is metabolically active. It will be understood that in this context, the term "conditions" includes the temperature, pH, appropriate composition of the substrate material or presence/absence of an inducer substance, at which the metabolic activity of the bacterial culture is optimal.

Bacteriophages require hosts with intact DNA replication, RNA transcription and protein synthesis in order to become proliferated. Accordingly, bacterial cultures used in the method of the invention are incapable of performing one of the above activities, which makes such bacterial cultures substantially completely resistant to attack by bacteriophages. Thus, the metabolic activity of the bacterial culture is substantially unaffected although the substrate material is contaminated with bacteriophages. As used herein, the expression "substantially unaffected" indicates that by using conventional detection methods no changes or only minor changes in the metabolic activity can be detected.

It will be appreciated that such auxotrophic bacteria can be provided by subjecting a wild type bacterial strain that, under appropriate conditions, is capable of growing in a substrate material with or without a specific compound needed for replication or protein synthesis to a mutagenization treatment and selecting a strain that is substantially incapable of growing in the absence of said specific compound.

Suitable mutagens include conventional chemical mutagens and UV light. Thus, as examples, a chemical mutagen can be selected from (i) a mutagen that associates with or becomes incorporated into DNA such as a base analogue, e.g. 2-aminopurine or an interchelating agent such as ICR-191, (ii) a mutagen that reacts with the DNA including alkylating agents such as nitrosoguanidine or hydroxylamine, or ethane methyl sulphamate (EMS).

As an alternative, auxotrophic bacteria can be provided by selecting spontaneously occurring mutants which, compared to the parent strain, has a growth requirement for a compound needed for DNA replication, RNA transcription or protein synthesis. It will be understood that it is also possible to provide an auxotrophic mutant by site-directed mutagenesis, e.g. by using recombinant DNA techniques.

The above bacterial wild type parent strain can be selected from any industrially suitable bacterial species, i.e. the strain can be selected from the group consisting of *Lactococcus* spp. including *L. lactis*, *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Actinomycetes* spp., *Enterobacteriaceae* spp. including *E. coli*, *Corynebacterium* spp. and *Brevibacterium* spp.

It is convenient to provide the above bacterial strains both when used as a food or feed production strain and as a pro-

duction strain for metabolites, as a composition comprising the bacterial strain selected for the specific use. Typically, such compositions contain the bacterium in concentrated form e.g. at a concentration of viable cells (colony forming units, CFUs) which is in the range of 10^5 to 10^{13} per g of the composition such as in the range of 10^6 to 10^{12} per g. Additionally, the culture composition may contain further components such as bacterial nutrients, cryoprotectants or other substances enhancing the viability of the bacterial active ingredient during storage. The composition can e.g. be in the form of a frozen or a freeze-dried composition.

In one specific embodiment of the method according to the invention, the *Lactococcus lactis* strain DN105 (DSM 12289) is used. This acidifying bacterial strain is a purine auxotrophic mutant capable of acidifying milk, even though the strain is not capable of growth because of its requirement for purine, which is not present in milk in sufficient amounts to support growth of such bacterium. As it is shown in the below Example strain DN105 is capable of acidifying milk under purine starvation conditions even in the presence of a high concentration of bacteriophages.

As mentioned above, one characteristic of the bacterial culture as used in the method of the invention, is that it is capable of metabolically modifying a specific defined substrate material even though the strain is incapable of growth in such substrate. When a lactic acid bacterium is used in the method of the invention, the obtained bacterial culture has an acidification rate in milk which is at least 10% of that of said culture when it is present in a substrate material where it is capable of replication and/or protein synthesis. In preferred embodiments, the bacterial strain has an acidification rate in milk which is at least 1% including at least 5%, such as at least 10%, e.g. at least 15%, such as at least 20%, including at least 25% of that of the culture when it is present in a substrate material where it is capable of replication and/or protein synthesis.

Typically, the bacterial strain used in the method of the invention is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material, such as at least 10^5 CFU/ml or g of the material, including
5 at least 10^6 CFU/ml or g of the material, such as at least 10^7 CFU/ml or g of the material, e.g. at least 10^8 CFU/ml or g of the material, including at least 10^9 CFU/ml or g of the material.

Thus, the obtainment of a bacterial culture which is completely resistant to bacteriophage attack according to the
10 method of the invention, can be utilized in all industrial contexts where proliferation of the culture in the substrate material is not a requirement. Dairy fermentations of milk is such an example of an industrial manufacturing process, as
15 proliferation of the lactic acid bacteria during milk fermentation is not a requirement, if the desired taste and acidification of the fermentation product are obtained. Therefore, in useful embodiments, the substrate material is a starting material for an edible product including milk, a
20 vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

In further embodiments, the substrate material is a starting material for an animal feed such as silage e.g. grass, cereal material, peas, alfalfa or sugar-beet leaf, where bacterial
25 cultures are inoculated in the feed crop to be ensiled in order to obtain a preservation hereof, or in protein rich animal waste products such as slaughtering offal and fish offal, also with the aims of preserving this offal for animal feeding purposes.

30 Yet another significant application of the method according to the present invention is the use of the bacterial cultures as so-called probiotics. By the term "probiotic" is in the present context understood a microbial culture which, when ingested in the form of viable cells by humans or animals,
35 confers an improved health condition, e.g. by suppressing

harmful microorganisms in the gastrointestinal tract, by enhancing the immune system or by contributing to the digestion of nutrients.

It is, as mentioned above, an important objective of the present invention to provide a method of preventing bacteriophage infection of bacterial cultures which are metabolically active by using non-proliferating bacterial cells. In order to be of industrial interest, such metabolic activity should result in the production of a substantial amount of the desired end product. Thus, one possibility of increasing such production by use of a non-proliferating cell is an enhancement of the flux through metabolic pathways.

Accordingly, in one useful embodiment, the bacterial culture used in the method of the invention comprises a genetically modified strain which, relative to its parent strain, is enhanced in at least one metabolic pathway. Such enhanced metabolic activity can e.g. be obtained through an enhanced glycolytic pathway and/or an enhanced flux through the pentose phosphate pathway.

One approach for stimulating the flux through the glycolytic pathway is by increasing the expression of ATPase activity, i.e. an enhanced conversion of ATP to ADP, as described in WO 98/10089. Thus, in one useful embodiment of the invention, the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

In one interesting embodiment of the present invention, the genetically modified strain is one wherein the gene coding for an ATPase is under the control of a regulatable promoter. As used herein, the term "regulatable promoter" is used to describe a promoter sequence possibly including regulatory sequences for the promoter, which promoter is regulatable by one or more factors present in the environment of the strain. Such factors include the pH of the growth medium, the growth temperature, a temperature shift eliciting the expression of

heat shock genes, the composition of the growth medium including the ionic strength/NaCl content and the growth phase/growth rate of the bacterium. Such a regulatable promoter may be the native promoter or it may be an inserted
5 promoter not naturally related to the gene either isolated from the same bacterial species or it may be a heterologous promoter sequence, i.e. a sequence derived from different bacterial species.

Cells such as "resting cells" or "non-dividing cells" represent other types of non-proliferating cells which are useful
10 in the method of the invention and wherein the above enhancement of the flux through the metabolic pathways is useful. Such cells are incapable of mitosis or meiosis e.g. due to the deficiency of DNA, RNA and/or protein needed for the
15 separation of the cell. Thus, in a particularly useful embodiment, the bacterial culture is one which comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

In addition, the invention encompasses non-proliferating
20 strains which under specific conditions are incapable of growth. Thus, in an interesting embodiment, the bacterial culture comprises a strain which is a conditional mutant, i.e. a mutant which under predetermined conditions does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein
25 synthesis. Such predetermined conditions include pH, temperature, composition of the substrate material and presence/absence of inducer substances.

One possible means of providing such conditional mutants
30 which are temperature-sensitive is by subjecting a bacterial strain that under appropriate conditions is capable of growing in a substrate material e.g. at a temperature below 30°C to a mutagenization treatment and selecting a mutant strain that substantially is incapable of growth at temperatures
35 below 30°C.

In further aspects, the invention relates to the use of a culture as obtained in the method according to the invention as a starter culture in the preparation of a product selected from the group consisting of a starter distillate, a product
5 for cheese flavouring, a food product and a feed product.

The invention is further illustrated in the following examples and the drawing wherein:

Fig. 1. shows the acidification of RSM by strain DN105. The pH was followed over time in RSM cultures containing strain
10 DN105 inoculated at 1%, 10%, 25% and 50% vol/vol,

Fig. 2 illustrates the development of pH in RSM when inoculated with 25% vol/vol of strain CHCC373 or strain DN105, in the presence of the bacteriophage strain 836 at a concentration of at least 10^8 PFU/ml, and

15 Fig. 3A-3H illustrate the development of pH in RSM when inoculated with 25% vol/vol of strain DN105 with or without addition of the purine compound hypoxanthine to the culture medium, in the presence of at least 10^8 PFU/ml of the following bacteriophages: CHPC836 (3A); CHPC412 (3B); CHPC783 (3C);
20 CHPC795 (3D); CHPC710 (3E); CHPC12 (3F); CHPC708 (3G); CHPC814 (3H).

EXAMPLE 1

The use of a purine auxotrophic *Lactococcus lactis* strain for obtaining resistance against bacteriophages in milk fermenta-
25 tions

1.1. Materials and methods

(i) Bacterial strains, media and growth conditions

The *Lactococcus lactis* strain DN105 pur is a purine auxotrophic mutant derived from the wildtype strain CHCC373 described

in Nilsson and Lauridsen (1992) and deposited with Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 30 June 1998 under the accession No. DSM 12289. *L. lactis* was grown in M17
5 (Terzaghi & Sandine, 1975) in the chemically defined DN medium (Dickely et al., 1995) or in reconstituted skim milk (RSM) containing 9.5% (w/vol) low fat skim milk powder at 30°C. For the propagation of strain DN105, the medium was supplemented with hypoxanthine to a final concentration of
10 15-50 mg/l. The Pur⁻ phenotype of strain DN105 was tested by its ability to grow on DN medium with and without hypoxanthine supplement.

(ii) Bacteriophages and their handling

Bacteriophages were purified by three single plaque isolation
15 steps. The high titer bacteriophage lysates were prepared by consecutive infections of the host strain CHCC373 with the bacteriophage at an MOI (multiplicity of infection) of 0.1 to 1.0. After infection the culture was grown at 30°C in M17 supplemented with 10 mM CaCl₂ until completed lysis. The
20 lysates were centrifuged for 15 min at 6.000 rpm and the supernatant sterile filtered (0.45 µm, Schleicher und Schuell).

(iii) Determination of bacteriophage titers

For the determination of bacteriophage titers (plaque forming
25 units per ml) the agar double layer method was used (Adams M.H., 1959; Interscience Publishers, Inc., New York). The bacteriophages used for the acidification test are listed in Table 1 below:

Table 1. Bacteriophages and phage titers

Phage	Titer (PFU/ml)
CHPC12	4×10^{10}
CHPC412	3×10^{10}
CHPC708	2×10^{10}
CHPC710	2×10^{10}
CHPC783	1×10^{11}
CHPC795	7×10^{10}
CHPC814	1×10^{10}
CHPC836	4×10^{10}

(iv) Test for the acidification of RSM by strain DN105

10-400 ml of an outgrown culture of strain DN105 in M17 was harvested by centrifugation, washed twice with a sterile solution of 0.9% NaCl to remove residual purine compounds and resuspended in 10-400 ml of RSM to give the same cell density as in the outgrown M17 culture. The resuspended material was used for inoculation of fresh RSM at volume/volume concentrations typically in the range of 10 to 100% (v/v). The pH was monitored either on-line or by measuring the pH of 3 ml samples collected at intervals.

1.3. Results of the acidification of RSM by *Lactococcus lactis* strain DN105 pur

In general, lactic acid bacterial cells that do not have intact DNA replication, RNA transcription and protein synthesis systems are unable to grow and acidify milk. Nilsson and Lauridsen (1992) demonstrated that the purine auxotrophic mutant DN105 is unable to grow in a medium without purines. It has also been reported that purine auxotrophic mutants of *Lactococcus lactis* not growing in milk are incapable of acidifying such a substrate material (Dickely et al., 1995). To test the ability of strain DN105 to possibly acidify a

milk based medium, the strain was inoculated in varying amounts in RSM as described in Materials and Methods and the pH of the substrate material was monitored (Fig. 1).

The results shown in Fig. 1 clearly demonstrates that strain
5 DN105 was able to acidify milk at least to pH 5.0 even under purine starving conditions.

1.4 Studies of the bacteriophage resistance of *Lactococcus lactis* strain DN105 pur in RSM

As it is generally known, a bacteriophage requires a suscep-
10 tible host cell which has intact DNA replication, RNA trans-
cription and protein synthesis systems for development.
If a potential host is not capable of performing at least one
of these activities, bacteriophages cannot be proliferated in
the cell.

15 Fig. 2 shows the development of pH in RSM when inoculated
with 25% vol/vol of the wildtype strain CHCC373 or the mutant
strain DN105 in the presence of the bacteriophage CHPC836 at
a concentration of at least 10^8 PFU/ml.

In further experiments the resistance of strain DN105 against
20 various bacteriophages was studied in RSM with and without
hypoxanthine added. The bacteriophages were added to RSM at
 10^8 to 10^9 bacteriophages/ml.

The addition of hypoxanthine was used as a positive control
for the bacteriophage infection, as the addition of this
25 purine compound enabled bacteriophage attack (Fig. 3 A-H). In
all cultures without hypoxanthine added strain DN105 acid-
ified the milk to around pH 5.0, whereas with the addition of
hypoxanthine none of the cultures reached a pH of below 5.4.

1.5. Discussion

This Example shows that the starvation of a *L. lactis pur*⁻ strain for purines causes total resistance to a range of bacteriophages and that the *L. lactis pur*⁻ strain is capable
5 of effectively acidifying milk in the presence of a large number of bacteriophages for which the corresponding wildtype is susceptible.

Thus, the present Example shows that it is possible to develop strains which under appropriate selected conditions, where
10 the strains are incapable of growth, are completely resistant to bacteriophages and that such strains has retained the metabolic ability to acidify milk. Such a system of bacteriophage resistance can be introduced into any bacteria e.g. *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp.,
15 *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Actinomyces* spp., *Enterobacteriaceae* spp. including *E. coli*, *Corynebacterium* spp. and *Brevibacterium* spp in regard to e.g. production of a bacterial fermentation
20 product such as lactate, diacetyl, acetoin, methanethiol, ethanol etc. if proliferation of the bacteria is not a requirement.

REFERENCES

1. Dickely, F., D. Nilsson, E. B. Hansen, and E. Johansen.
1995. Isolation of *Lactococcus lactis* nonsense suppressors
and construction of a food-grade cloning vector. *Mol. Micro-*
5 *biol.* 15:839-847.
2. Nilsson, D., and A. A. Lauridsen. 1992. Isolation of
purine auxotrophic mutants of *Lactococcus lactis* and
characterisation of the gene *hpt* encoding hypoxanthine
guanine phosphoribosyltransferase. *Mol. Gen. Genet.* 235:359-
10 364.
3. Richardson, G.H., Gamay, A.Y, Shelaih, M.A., Kim, J.M. and
Hansen, C.L. 1984. Paired and single strain protease negative
lactic *Streptococci* for cheese manufacturing. *J. Dairy Sci.*
67:518-521.
- 15 4. Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium
for lactic streptococci and their bacteriophages. *Appl.*
Microbiol. 29:807-813.

CLAIMS

1. A method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of replication or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,

(ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the material under conditions where the culture is metabolically active.

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

2. A method according to claim 1 wherein the substrate material is limited with respect to at least one compound that is required by the bacterial strain for replication or protein synthesis.

3. A method according to claim 2 wherein the bacterial strain is a mutant strain being auxotrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

4. A method according to claim 3 wherein the mutant strain is a Pur⁻ mutant including *Lactococcus lactis* strain DN105 deposited under the accession number DSM 12289.

5. A method according to any of claims 2 to 4 wherein the strain in said substrate material is not capable of performing at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.
- 5 6. A method according to claim 1 wherein the substrate material contains at least one compound that inhibits the replication or the protein synthesis of the bacterial strain.
7. A method according to claim 1 wherein the substrate material is a starting material for an edible product, the
10 material is selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.
8. A method according to claim 1 wherein the bacterial culture is selected from the group consisting of *Lactococcus*
15 spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli*, *Actinomycetes* spp., *Corynebacterium* spp. and *Brevibacterium* spp.
- 20 9. A method according to claim 8 wherein the bacterial culture is of *Lactococcus lactis*.
10. A method according to claim 1 wherein the bacterial strain is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material.
- 25 11. A method according to claim 1 wherein the culture comprises a genetically modified strain which, relative to its parent strain is enhanced in at least one metabolic pathway.
12. A method according to claim 11 wherein the genetically modified strain has, relative to its parent strain, an
30 enhanced metabolic activity selected from the group consist-

ing of enhanced glycolytic flux and enhanced flux through the pentose phosphate pathway.

13. A method according to claim 12 wherein the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

14. A method according to claim 1 wherein the bacterial culture comprises a strain which is a conditional mutant which at a predetermined condition does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

15. A method according to claim 14 wherein the predetermined condition is selected from the group consisting of pH, temperature, composition of the substrate material and presence/absence of an inducer substance.

16. A method according to claim 1 wherein the culture comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

17. Use of a culture as obtained in the method of claim 1 as a starter culture in the preparation of a product selected from the group consisting of a starter distillate, a product for cheese flavouring, a food product and a feed product.

ABSTRACT

Method of preventing bacteriophage infection of bacterial cultures comprising modified strains, wherein the cultures are completely resistant to bacteriophage attack and have
5 retained their capability of being metabolically active. The method is useful in the manufacturing of food products, feed products or useful metabolite products.

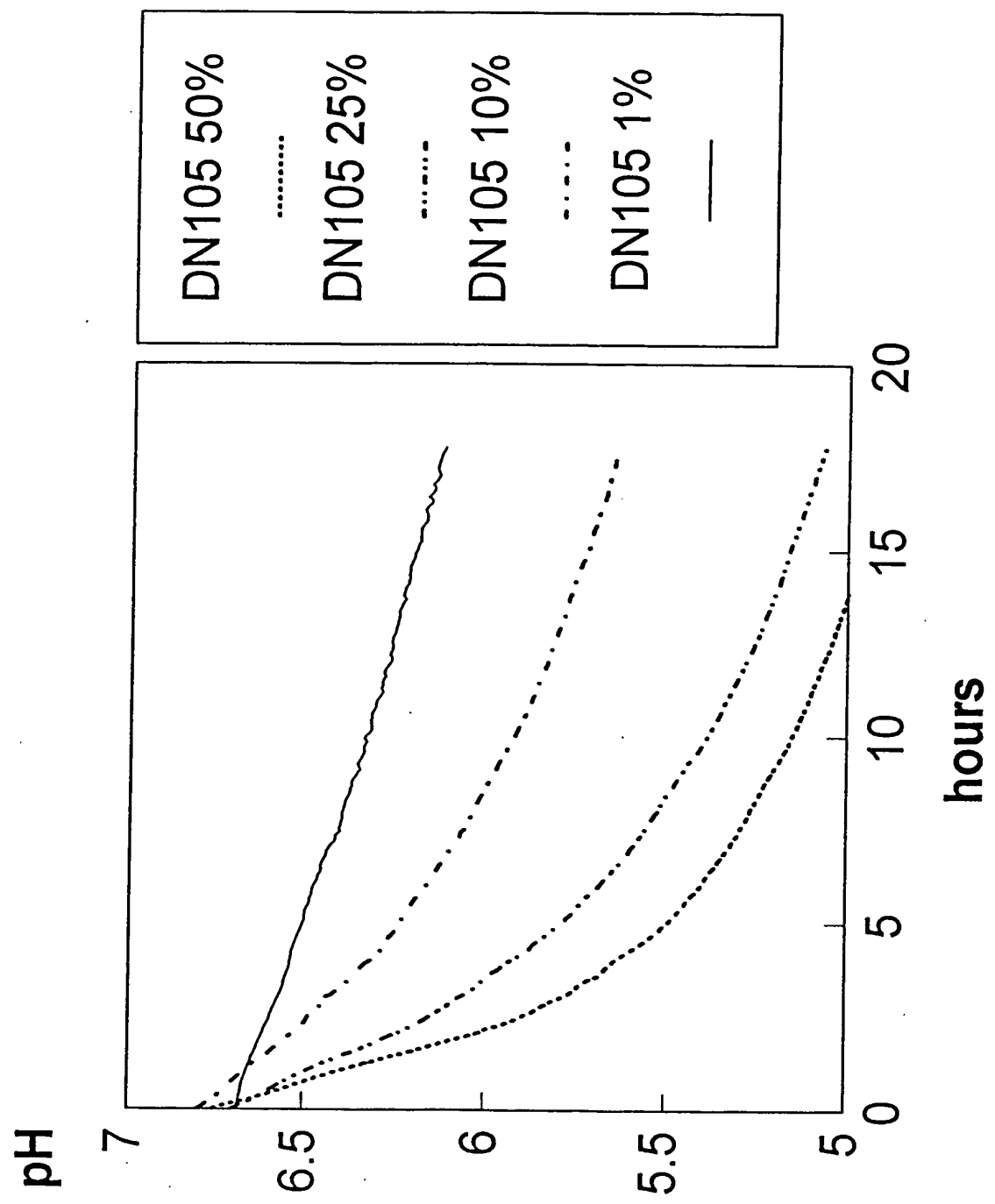


Fig. 1

- 3 JULI 1998

2/10

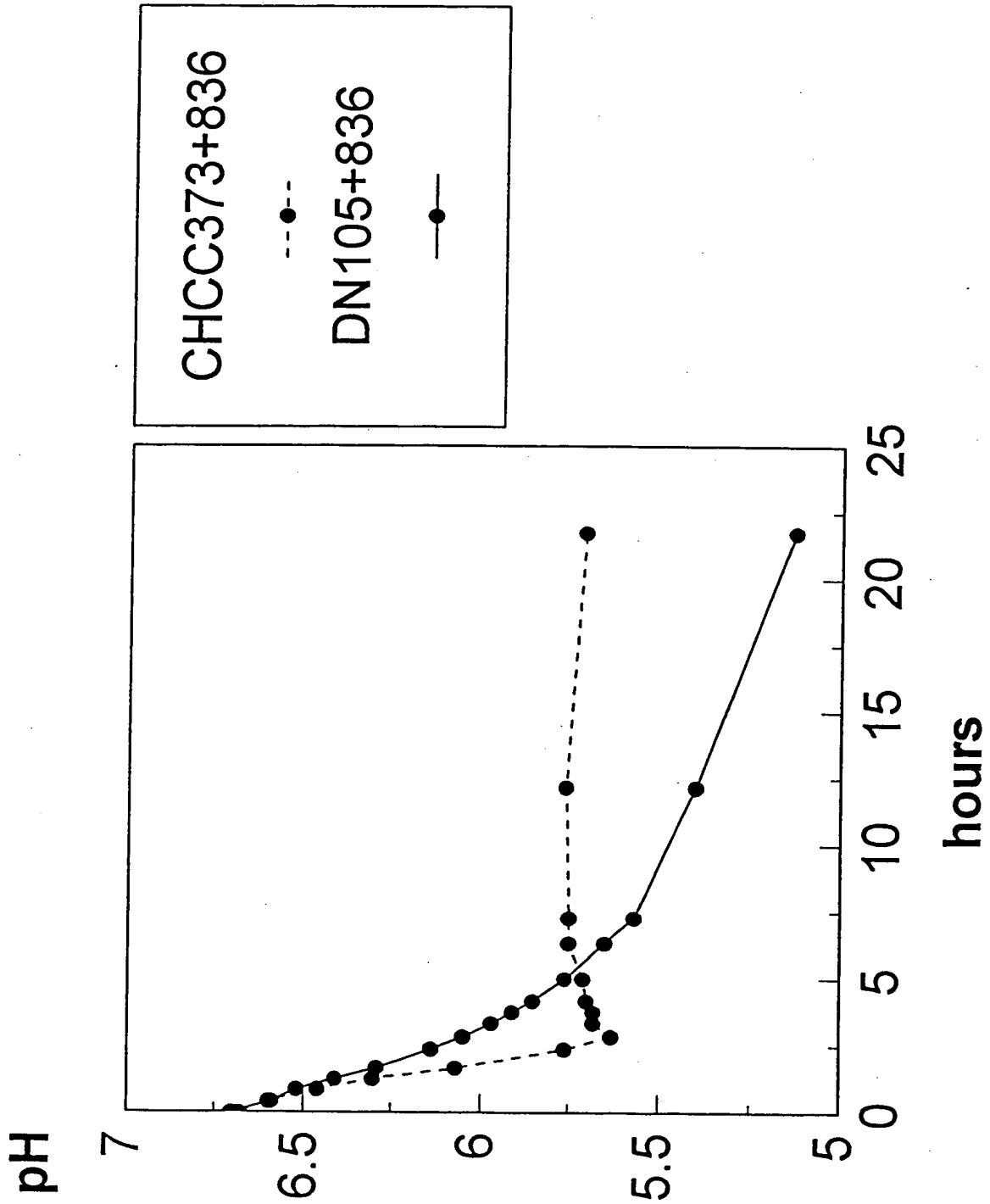


Fig. 2

3/10

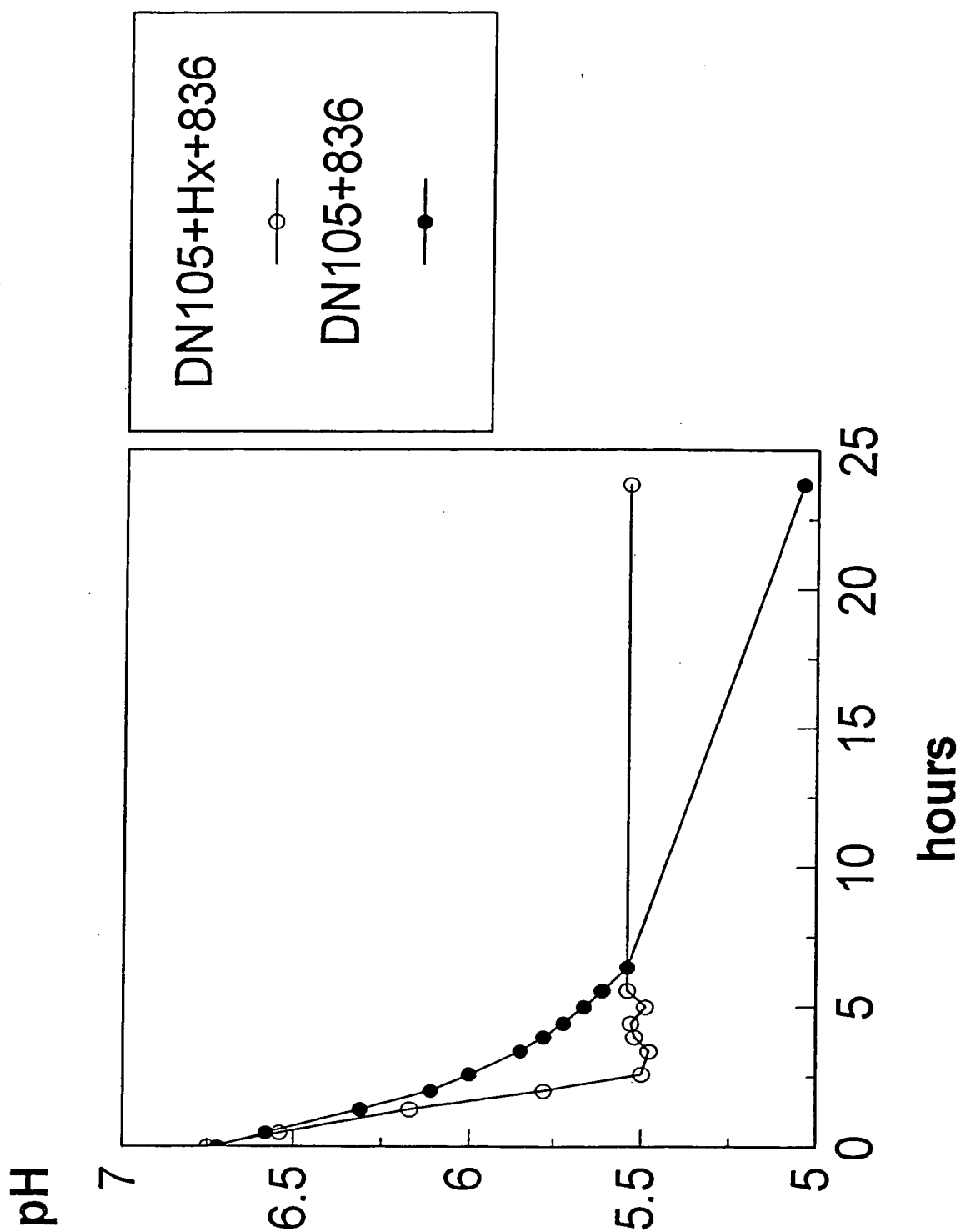


Fig. 3A

4/10

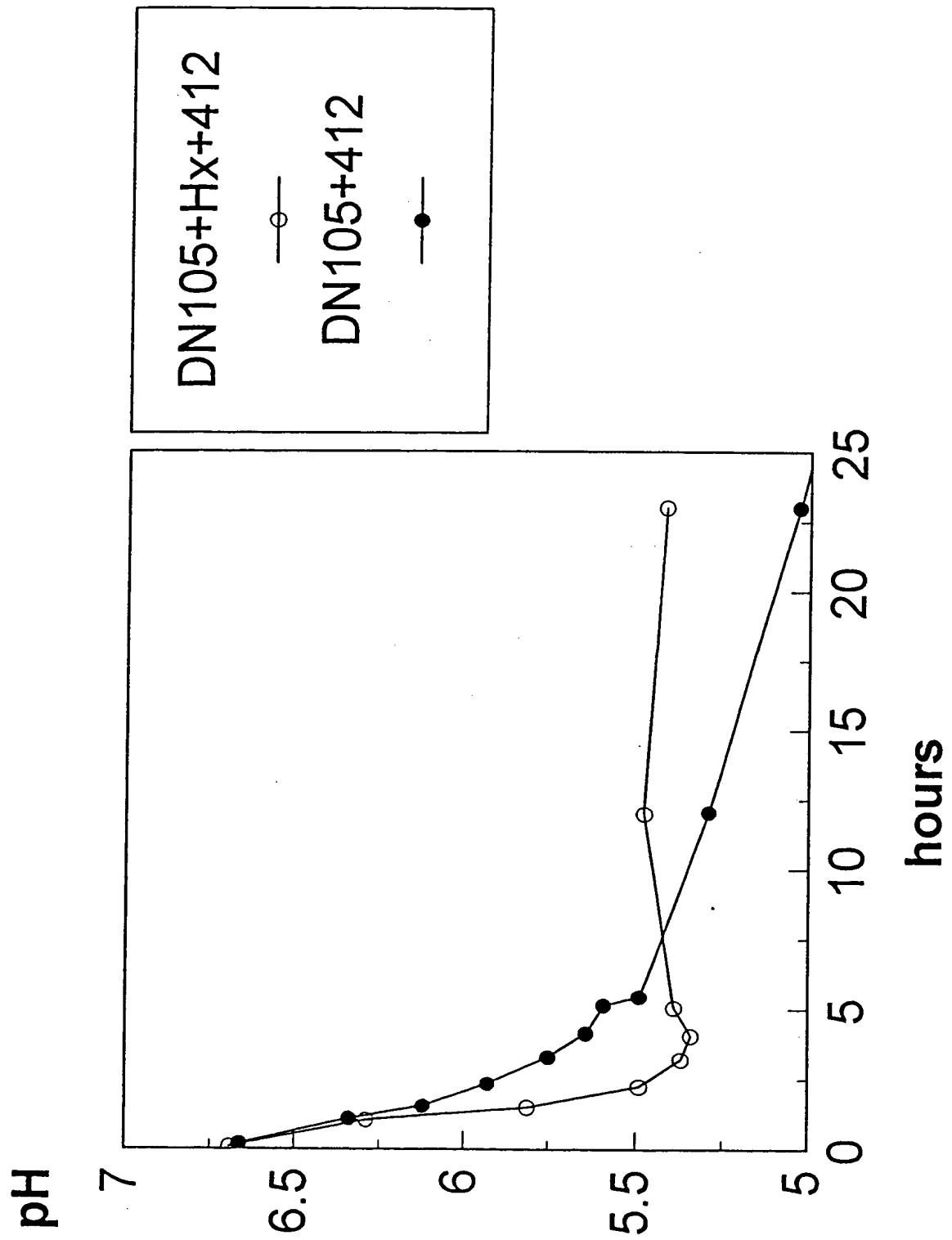


Fig. 3B

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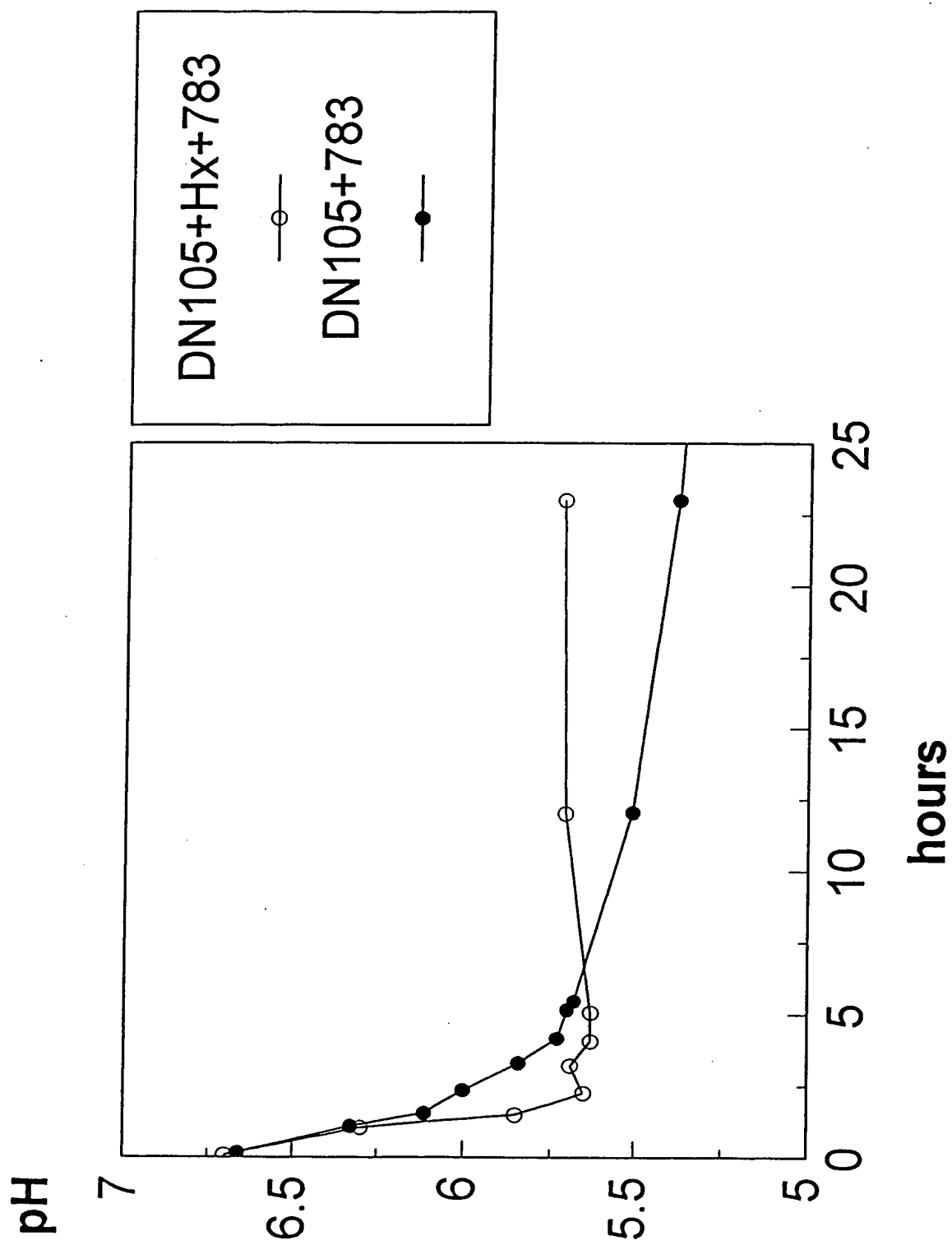


Fig. 3C

- 3 JULI 1998

6/10

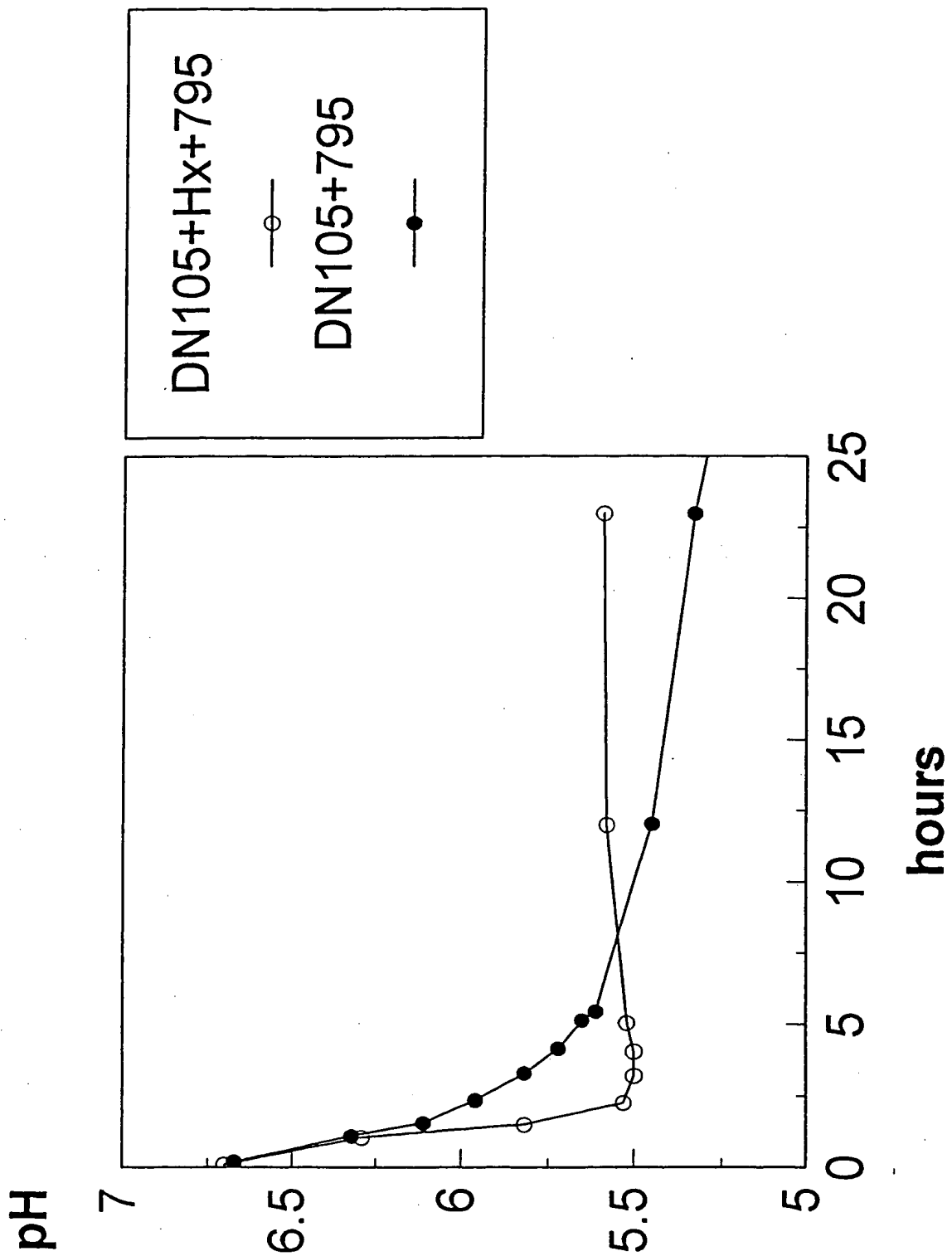


Fig. 3D

7/10

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- 3 JULI 1998

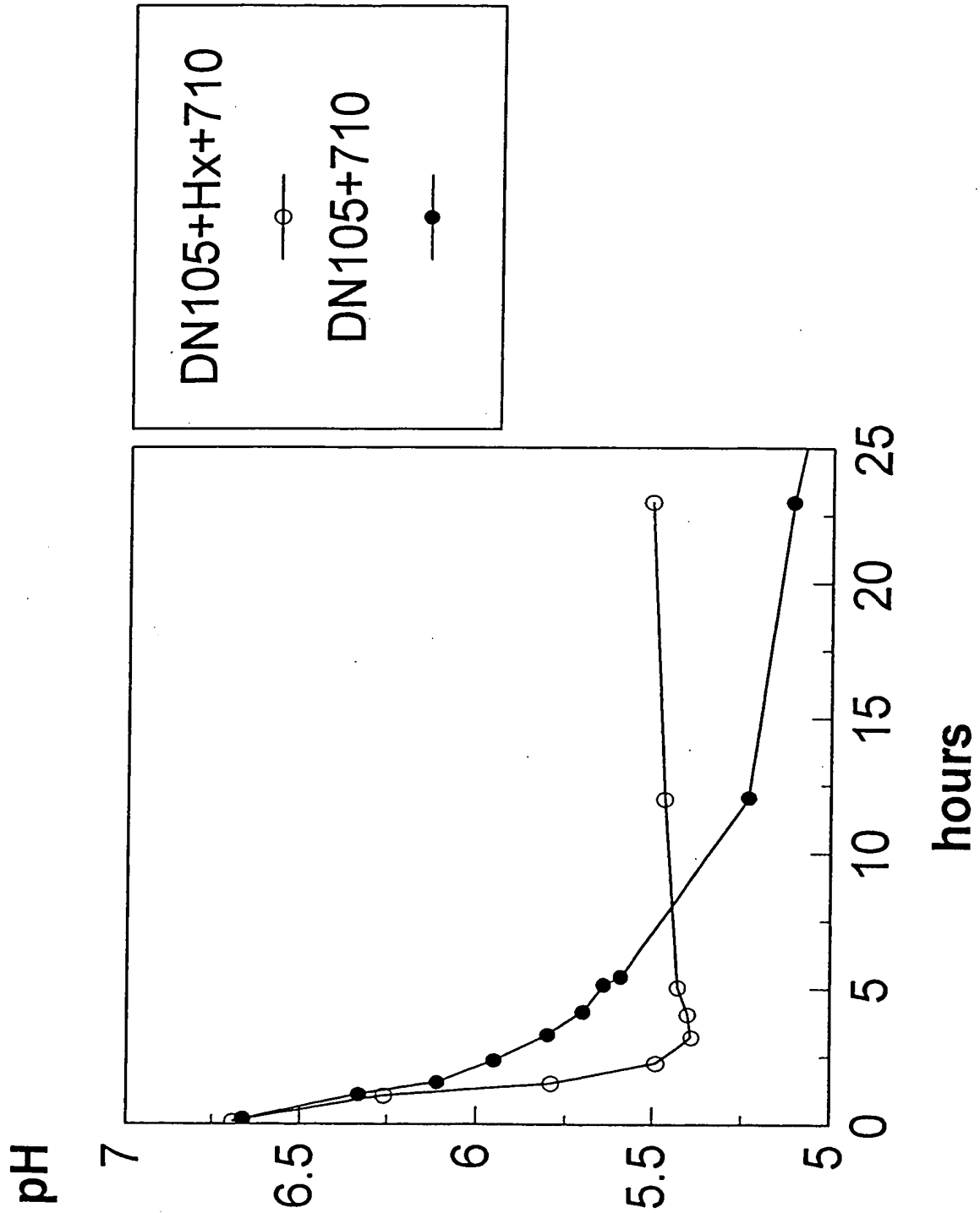


Fig. 3E

8/10

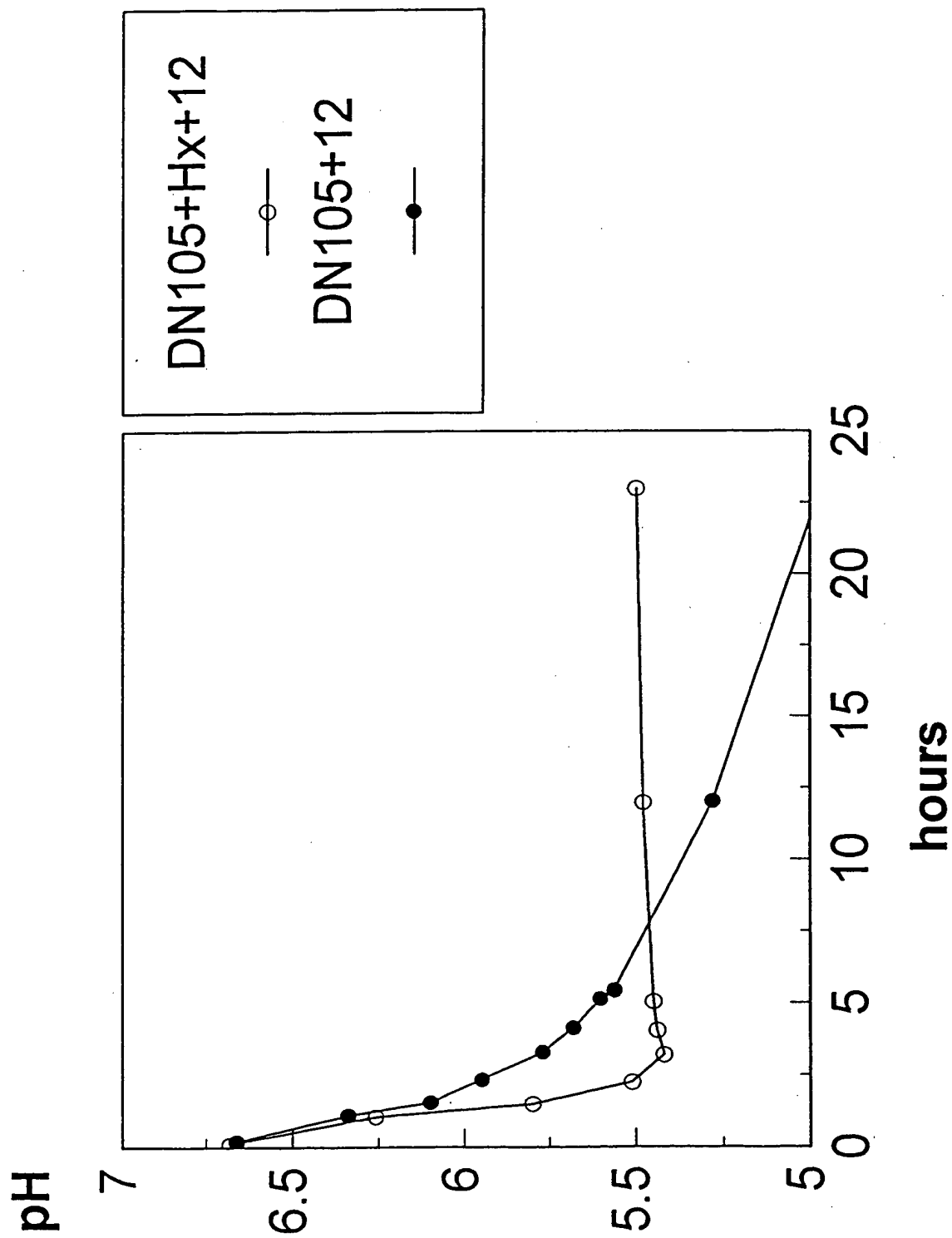


Fig. 3F

- 3 JULI 1998

9/10

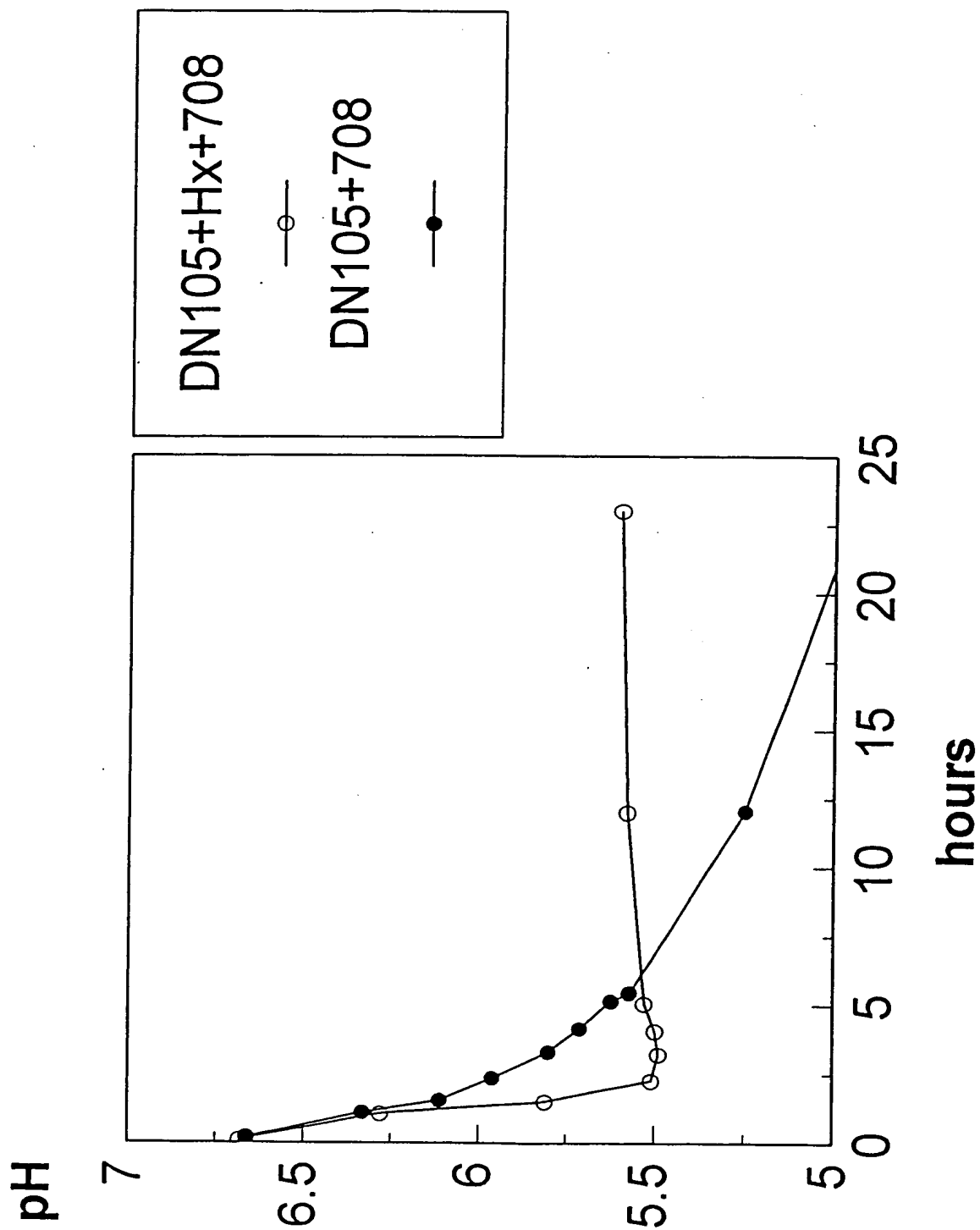


Fig. 3G

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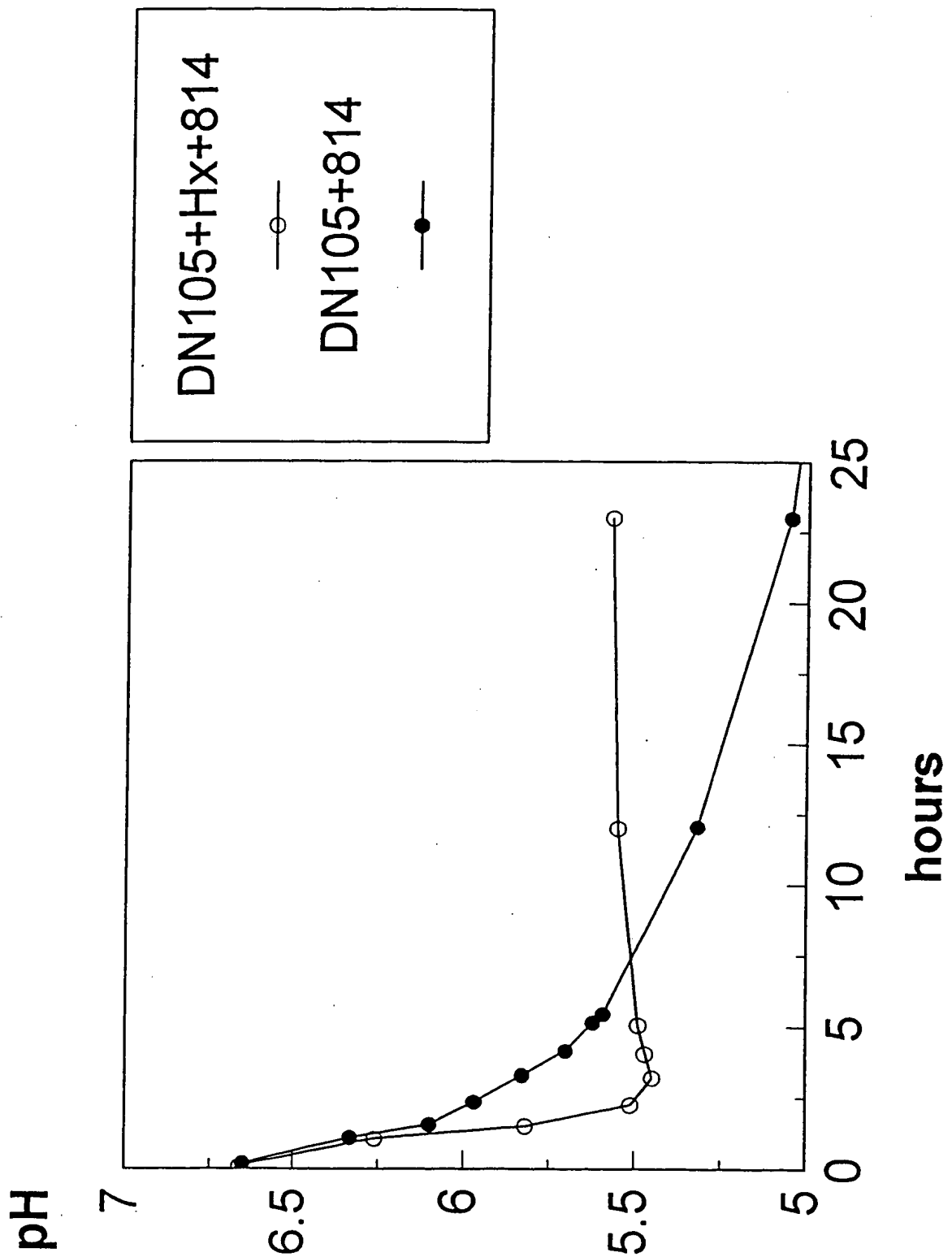


Fig. 3H